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Inactivation of human norovirus surrogate, human norovirus virus-like particle, and vesicular stomatitis virus by gamma irradiation: sensitivity and mechanism.

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Abstract

Gamma irradiation is a non-thermal processing technology that has been used for preservation of varieties of food products. This technology has been shown to effectively inactivate bacterial pathogens. Currently, the FDA has approved doses of up to 4.0 kGy to control foodborne pathogens in fresh iceberg lettuce and spinach. However, whether this dose range effectively inactivates foodborne viruses is less understood. We have performed a systematic study on the inactivation of human norovirus surrogate (murine norovirus 1, MNV-1), human norovirus virus-like particles (VLPs), and vesicular stomatitis virus (VSV) by gamma irradiation. We demonstrated that MNV-1 and human norovirus VLPs were resistant to gamma irradiation. For MNV-1, only 1.7-2.4 log virus reduction was observed in fresh produce at the dose of 5.6 kGy. However, VSV was more susceptible to gamma irradiation, and a 3.3 log virus reduction was achieved at a dose of 5.6 kGy in DMEM. We further demonstrated that gamma irradiation disrupted virion structure, and degraded viral proteins and genomic RNA which resulted in virus inactivation. Using human norovirus VLPs as a model, we provided first evidence that the capsid of human norovirus has a similar stability compared to MNV-1 after exposure to gamma irradiation. Overall, our results suggest that viruses are much more resistant to irradiation than bacterial pathogens. Although gamma irradiation seems impractical to eliminate the virus contaminants in fresh produce by the FDA approved irradiation dose limits, this technology may be practical to inactivate viruses for other purposes such as sterilization of medical equipment.

53 ## Introduction

54

55 Norovirus is a major food and waterborne pathogen that causes acute gastroenteritis in
56 humans. The virus is primarily transmitted through the fecal-oral route, either by direct contact
57 with fecal matter or indirect contact through contaminated food or water supplies. It is highly
58 stable and contagious, requiring less than ten virus particles to cause an infection (20, 35, 44,
59 51). Recent human volunteer study and mathematical modeling showed that the average
60 probability of infection for a single norovirus particle to be close to 0.5 (56). Norovirus
61 outbreaks occur worldwide and have a significant impact in food safety and public health. The
62 National Institute of Allergy and Infectious Diseases (NIAID) categorized human norovirus as a
63 Category B priority bio-defense pathogen. Food associated outbreaks occur in diverse locales
64 including restaurants, nursing homes, schools, cruise ships, and personal residences (1, 15, 21,
65 25, 31, 43). In fact, it is estimated that over 90% of nonbacterial gastroenteritis is caused by
66 norovirus infections (21, 35, 43). This may even be an under estimation due to the large number
67 of unreported or asymptomatic infections (21, 35). The virus has a significant economic and
68 emotional impact in society; however research on this bio-defense agent has been impeded
69 because it cannot be cultivated *in vitro* (19).

70 Foods at high risk of norovirus contamination include fresh produce. Norovirus
71 contamination can occur during pre-harvest and post-harvest stages, such as by contaminated
72 irrigation water, septic tank runoff, and mishandling by farm workers and food handlers (2, 8, 24,
73 26, 39, 53). Additionally, fresh produce undergoes minimal to no processing and thus increasing
74 the viral infection risk (8, 18, 26, 54). In fact, norovirus accounts for more than 40% of fresh
75 produce associated outbreaks (18). Berries shipped across international borders have also been

known to cause outbreaks because the virus can remain stable on the frozen tissue (10, 11, 48). Currently, chlorine-based sanitizers are widely used in the fresh produce industry as the primary decontamination method for pathogen removal (3, 18). However, it has been demonstrated that these sanitizers are not effective in removing human norovirus surrogates and hepatitis A virus from fresh produce (3, 53). Chlorine solution rinses can only achieve a maximum 1-1.5 log virus reduction in contaminated fresh produce (3, 53). More importantly, recent evidence suggests noroviruses can become internalized in fresh produce (50, 61, 62). Chemical sanitizers may reduce the viruses at the surface level of produce, but not those that have been internalized.

One potential technology to decontaminate the pathogens in fresh produce is irradiation technology (22, 59). High frequency electromagnetic waves (gamma rays) are released during isotopic negative beta decay. The decay involves the conversion of a neutron into a proton which releases an electron and an antineutrino; gamma rays are released during this process (17, 23). Gamma ray frequencies are in the magnitude of 10^{19}Hz; these high energy electromagnetic waves may hold potential in disrupting viral pathogens (17, 23). The high energy waves can penetrate into food products, and thus can potentially inactivate internalized pathogens. Currently, the FDA has approved doses of up to 4.0 kGy to control foodborne pathogens in fresh iceberg lettuce and spinach (FDA Regulations, 21 CFR 179.26). It has been shown that gamma irradiation effectively inactivated *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* in food products, including fresh produce, at this approved dose (5, 17, 34, 45, 59).

However, the feasibility of using gamma irradiation to eliminate human norovirus, the major causative agent of foodborne illnesses, is not understood. This is due to the fact that human norovirus lacks a cell culture system (19). Thus, survival studies of human norovirus

must rely on suitable surrogates such as feline calicivirus (FCV) (16, 60), canine calicivirus (CaCV) (16), and murine norovirus (MNV-1) (4, 13). It was reported that a 3 log virus reduction was observed in low protein-content solution at 0.5kGy for FCV and 0.3 kGy for CaCV by gamma irradiation (16). It seems that these two surrogates were quite susceptible to gamma irradiation, or at least in low-protein-content solutions. However, no survival trend data have been reported for any human norovirus surrogates in food samples including fresh produce. More importantly, recent studies found that MNV-1 is a better surrogate for human norovirus due to their biochemical, genetic, and morphological similarities (13). For example, the size (35-38 nm in diameter), shape (icosahedral, small rounded structure), viral capsid, and RNA genome of MNV-1 are all analogous to human norovirus (32, 63). Furthermore, MNV-1 is highly resistant to acidic environments and is more stable than FCV (13). To date, no study has been performed investigating the susceptibility of MNV-1 to gamma irradiation. Moreover, the mechanism of viral inactivation by irradiation is poorly understood. Noroviruses and other caliciviruses are non-enveloped viruses and the outer shell of these viruses possesses a highly stable capsid that protects their genetic material, the single-stranded positive-sense RNA. The effect of gamma irradiation on viral capsid and genomic RNA has not been determined.

In this study, we performed a systematic study on the inactivation of MNV-1 and human norovirus virus-like particles (VLPs) by gamma irradiation. We also selected vesicular stomatitis virus (VSV), an enveloped virus, as a comparison since the virion structure of VSV is significantly different compared to norovirus. Furthermore, we determined the mechanism of viral inactivation by gamma irradiation. We investigated the effect of gamma irradiation on viral proteins, genomic RNA, capsid integrity, and virus particles. In addition, we provided first

120 evidence that the capsid proteins of human norovirus VLPs and MNV-1 have a similar stability
121 after exposure to gamma irradiation.

122

123 **Materials and Methods**

124 **Viruses and cell culture.** Murine norovirus strain MNV-1 was generously provided by Dr.
125 Herbert W. Virgin IV, Washington University School of Medicine (32). Vesicular stomatitis
126 virus (VSV) Indiana strain was a generous gift from Dr. Sean Whelan at Harvard Medical School
127 (36, 37). MNV-1 and VSV were propagated in confluent monolayers of murine macrophage
128 cell line RAW 264.7 (ATCC, Manassas, VA) and baby hamster kidney cells (BHK-21 cells),
129 respectively. Both RAW 264.7 and BHK-21 cells were cultured in high-glucose Dulbecco's
130 modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal
131 bovine serum (FBS) (Invitrogen), at 37 ^{o}C under a 5% CO_2 atmosphere. For growing MNV-1
132 stock, confluent RAW 264.7 cells were infected with MNV-1 at a multiplicity of infection (MOI)
133 of 0.01. After 1 h incubation at 37 ^{o}C, 15 ml of DMEM supplemented with 2% FBS was added.
134 The virus was harvested after 2 days post inoculation by three freeze-thaw cycles and low speed
135 centrifugation at 15,000 rpm for 30 min. Preparation of VSV stock was performed as described
136 previously. Briefly, confluent BHK-21 cells were infected with VSV at a MOI of 3. After 1 h
137 incubation at 37 ^{o}C, 15 ml of DMEM supplemented with 2% FBS was added. Virus was
138 harvested after 18 h post inoculation by low speed centrifugation at 5,000 rpm for 10 min.

139

140 **Sample preparation and gamma irradiation.** Fresh produce (strawberry, romaine lettuce, and
141 spinach) was purchased from a local supermarket. One ml of MNV-1 virus stock (10^8 PFU/ml)

142 was inoculated into 10 g of each produce to achieve an inoculation level of 10^7 PFU/g. Samples
143 were prepared in triplicates. The samples were heat sealed in plastic compartments using an AIE-
144 200 Impulse Sealer (American International Electric, Whittier, CA) and mixed thoroughly on a
145 shaker for 30 min. All samples were stored in an ice box and delivered to the Nuclear Reactor
146 Laboratory (NRL) at The Ohio State University. Irradiation treatment took place in a 6 in
147 diameter cylinder in the NRL Cobalt-60 irradiator. Samples were irradiated at doses of
148 0.175kGy, 0.35kGy, 0.525kGy, 0.7kGy, 2.8kGy, 5.6kGy, 11.2kGy, 16.8kGy, and 22.4kGy.
149 After treatment, 5 ml of phosphate-buffered saline (PBS, 137 mM NaCl, 10 mM Na_2HPO_4, 2
150 mM NaH_2PO_4, pH 7.4) were added to each bag, and the samples were stomached for 2 min.
151 Virus was eluted by low speed centrifugation at 5,000 rpm for 10 min. The sensitivity of VSV
152 and MNV-1 to irradiation in DMEM was also tested. One ml of MNV-1 (10^8 PFU/ml) and VSV
153 (10^9 PFU/ml) stocks were inoculated into 10 ml of DMEM to achieve an inoculation level of 10^7
154 PFU/ml and 10^8 PFU/ml, respectively. To study the mechanism of viral inactivation, 20 µg of
155 highly purified MNV-1 and VSV (1 µg /µl) were prepared for gamma irradiation. All samples
156 were heat sealed in plastic compartments and irradiated for 2.8kGy, 5.6kGy, 11.2kGy, 16.8kGy,
157 and 22.4kGy. The viral survivors were determined by plaque assay.
158
159 **MNV-1 and VSV plaque assay.** MNV-1 and VSV plaque assay were performed in RAW 264.7
160 and Vero cells, respectively. Briefly, cells were seeded into six-well plates (Corning Life
161 Sciences, Wilkes-Barre, PA) at a density of 2×10^6 cells per well. After 24 h incubation, RAW
162 264.7 and Vero cell monolayers were infected with 400 µl of a 10-fold dilution series of MNV-1
163 or VSV, respectively, and the plates were incubated for 1 h at 37°C with agitation every 10 min.
164 The cells were overlaid with 3 ml of Eagle minimum essential medium (MEM) containing 1%

agarose, 2% FBS, 1% sodium bicarbonate, 0.1 mg of kanamycin/ml, 0.05 mg of gentamicin/ml , 15 mM HEPES (pH 7.7), and 2 mM L-glutamine. After incubation at 37 °C and 5% CO_2 for 24 h, the plates were fixed in 10% formaldehyde and the plaques were visualized by staining with 0.05% (w/v) crystal violet.

Purification of MNV-1 and VSV. To grow a large stock of MNV-1, 18 confluent T150 flasks of RAW 267.1 cells were infected with MNV-1 at a MOI of 0.01 in a volume of 3 ml of DMEM. At 1 h post-absorption, 15 ml of DMEM with 2% FBS was added to the flasks, and infected cells were incubated at 37°C for 48 h. When extensive cytopathic effect (CPE) was observed, cell culture fluid was harvested and subjected to three freeze-thaw cycles to release virus particles. The purification of MNV-1 was performed using the method described by Katpally et al (2008) with minor modifications (33). Briefly, virus suspension was centrifuged at 8,000×g for 15 min to remove cellular debris. The supernatant was digested with DNase I (10 µg/ml) and $MgCl_2$ (5 mM) at room temperature. After 1 h incubation, 10 mM EDTA and 1% lauryl sarcosine were added to stop nuclease activity. Virus was concentrated by centrifugation at 82,000×g for 6 h at 4°C in a Ty 50.2 rotor (Beckman, Brea, CA). The pellet was resuspended in PBS and further purified by centrifugation at 175,000 × g for 6 h at 4°C through a sucrose gradient (7.5 to 45%) in an SW55 Ti rotor (Beckman). The final virus-containing pellets were resuspended in 100 µl PBS. The virus titer was determined by plaque assay on RAW 264.7 cells. Viral protein was measured by Bradford reagent (Sigma Chemical Co., St. Louis, MO). The purified virus stock was diluted at the final concentration of 1 µg/µl. Approximately 1 mg of purified MNV-1 was obtained.

187 Purification of VSV was performed by the method described in our previous publication
188 (36, 37). Briefly, 10 confluent T150 flask BHK-21 cells were infected by VSV at a MOI of 0.01.
189 At 1 h post-absorption, 15 ml of DMEM (supplemented with 2% FBS) was added to the cultures,
190 and infected cells were incubated at 37°C. After 24 h post-infection, cell culture fluid was
191 harvested by centrifugation at 3,000 × *g* for 5 min. Virus was concentrated by centrifugation at
192 40,000 × *g* for 90 min at 4°C in a Ty 50.2 rotor. The pellet was resuspended in NTE buffer (100
193 mM NaCl, 10 mM Tris, 1 mM EDTA [pH 7.4]) and further purified through 10% sucrose NTE
194 by centrifugation at 150,000 × *g* for 1 h at 4°C in an SW50.1 rotor. The final pellet was
195 resuspended in 0.3 ml of NTE buffer. The virus titer was determined by plaque assay on Vero
196 cells, and the protein content was measured by Bradford reagent (Sigma Chemical Co.). The
197 purified virus stock was diluted at the final concentration of 1 µg/µl. Approximately 5 mg of
198 purified MNV-1 was obtained.

199

200 **Expression and purification of human norovirus virus-like particles (VLPs).** The capsid
201 VP1 gene of human norovirus G II.4 strain HS66 (GenBank accession no. EU105469, kindly
202 provided by Dr. Linda Saif, The Ohio State University) was amplified by high fidelity PCR, and
203 cloned into a pFastBac-Dual expression vector (Invitrogen) at *Sma* I and *Xho* I sites under the
204 control of the p10 promoter. The resultant plasmid pFastBac-Dual-VP1 was transformed into
205 DH10Bac competent cells. Baculovirus expressing VP1 protein was generated by transfection of
206 bacmids into *Spodoptera frugiperda* (Sf9) cells with a Cell-fectin Transfection kit (Invitrogen),
207 according to the instructions of the manufacturer. Purification of human norovirus virus-like
208 particles (VLPs) from insect cells was described in our previous publication (40). Briefly, Sf9
209 cells were infected with baculovirus at an MOI of 10, the infected Sf9 cells and cell culture

supernatants were harvested at 6 days post-inoculation. The VLPs were purified from cell culture supernatants and cell lysates by ultracentrifugation through a 40% (w/v) sucrose cushion, followed by CsCl isopycnic gradient (0.39 g/cm^3) ultracentrifugation. Purified VLPs were analyzed by SDS-PAGE, Western blot, and electron microscope (EM). The protein concentration of the VLPs was measured by Bradford reagent (Sigma Chemical Co.).

Reverse transcription polymerase chain reaction (RT-PCR). Viral genomic RNA was extracted from MNV-1 and VSV suspensions (either gamma irradiation treated or untreated) using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. 100 µl of unpurified virus suspension (10^8PFU/ml) or 10 µl of purified virus stock (total viral protein concentration at 1 µg/µl) was used for RNA extraction. RT-PCR was performed using a One Step RT-PCR kit (Qiagen). Two primers (5'-ATGAGGATGAGTGATGGCGC-3' and 5'-TTATTGTTTGAGCATTCGGCC-3') were designed to target the capsid MNV-1 VP1 gene. Two primers (5'-ATGTCTGTTACAGTCAAGAG-3' and 5'-TCATTTGTCAAATTCTGAC-3') were designed to target VSV N gene. All the primers were purchased from Sigma. One-step RT-PCR was performed in a 50 µl of reaction containing 400 µM of each dNTP, 0.6 µM of each primer, 4 µl of RNA template, 5 unit of RNase inhibitor, and 2 µl of RT-PCR Enzyme Mix. The amplified products were analyzed on 1% agarose gel electrophoresis.

Analysis of viral proteins by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A total of 20 µl of high purified viruses (viral protein concentration is1 µg/µl and viral titer is approximately 10^{11}PFU/ml) was treated by gamma irradiation. Two µg of highly purified MNV-1 and VSV suspensions (either gamma irradiation treated or untreated) were

233 analyzed by SDS-PAGE. Samples were boiled for 5 min in loading buffer containing 1% SDS,
234 2.5% β-mercaptoethanol, 6.25 mM Tris-HCl (pH 6.8) and 5% glycerol, and loaded into 12%
235 polyacrylamide gel. Viral proteins were visualized by Coomassie blue staining.
236
237 **Western Blotting.** MNV-1 and VSV virus proteins were separated by 12% SDS-PAGE and
238 transferred onto a Hybond ECL nitrocellulose membrane (Amersham, Piscataway, NJ) in a Mini
239 Trans-Blot electrophoretic transfer cell (Bio-Rad, Hercules, CA). For MNV-1, the blot was
240 probed with rabbit polyclonal MNV-1 antibody (a generous gift from Dr. Virgin) at a dilution of
241 1:10,000 in blocking buffer (5% skim milk), followed by horseradish peroxidase (HRP)-
242 conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA)
243 at a dilution of 1:20,000. Western blot of human norovirus VLPs was essentially identical to
244 MNV-1 VP1 with the exception of using polyclonal antibody against VP1protein of human
245 norovirus GII.4 strain (a generous gift from Dr. Xi Jiang, Cincinnati Children's Hospital Medical
246 Center). For VSV, the blot was probed with mouse monoclonal anti-VSV glycoprotein antibody
247 (Sigma-Aldrich) at a dilution of 1:5,000 in blocking buffer, followed by incubation with HRP-
248 conjugated anti-mouse IgG secondary antibody at a dilution of 1:100,000. Afterwards, the
249 membranes were washed three times with PBST + 0.02% Tween for 10 min each time. The blots
250 were developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific,
251 Pittsburg, PA) and exposed to Kodak BioMax MR film (Kodak, Rochester, NY).
252
253 **Transmission electron microscopy.** To determine whether gamma irradiation damages the
254 virus particles, negative staining electron microscopy of purified virions or human norovirus
255 VLPs was performed using the method described in our previous publications (38, 40). Briefly,

20 µl aliquots of either gamma irradiation treated or untreated samples were fixed in copper grids (Electron Microscopy Sciences, Inc., Hatfield, PA), and negatively stained with 1% ammonium molybdate. Virus particles were visualized by FEI Tecnai G2 Spirit Transmission Electron Microscope (TEM) at 80 kV at the Microscopy and Imaging Facility at The Ohio State University. Images were captured on a MegaView III side-mounted CCD camera (Soft Imaging System, Lakewood, CO) and figures were processed using Adobe Photoshop software (Adobe Systems, San Jose, CA).

Statistical analysis. All experiments were carried out in triplicate. Virus survival was expressed as mean log titer ± standard deviation. Quantitative analysis was performed by densitometric scanning of autoradiographs and ImageQuant TL software (GE Healthcare, Piscataway, NJ). Statistical analysis was performed by one-way multiple comparisons using SPSS 8.0 Statistical Analysis software (SPSS Inc., Chicago, IL, USA). A value of $p<0.05$ was considered statistically significant.

Results

Gamma irradiation inactivation of MNV-1 in fresh produce. Using cultivable murine norovirus as a surrogate, we have now systematically investigated the survival of the virus in fresh produce after gamma irradiation treatment. As shown in Fig.1, MNV-1 was gradually inactivated in fresh produce in a dose-dependent manner. However, MNV-1 is resistant to gamma irradiation. At low irradiation doses (0.175kGy, 0.35kGy, 0.525kGy, and 0.7kGy), there was no significant virus reduction (less than 0.28 log) in all fresh produce samples ($P>0.05$). At

dose of 2.8 kGy, only 1.77 log, 1.40 log, and 1.31 log reductions were observed in spinach, romaine lettuce, and strawberries, respectively. At dose of 5.6 kGy, only 1.7-2.4 log reduction was observed. Virus inactivation was significantly enhanced at a high dose of gamma irradiation ($P<0.05$). At a dose of 11.2 kGy, 3.6-4.1 log virus reductions were achieved in all fresh produce samples. At a dose of 22.4 kGy, MNV-1 was completely inactivated in romaine lettuce and strawberry, while 2.4 logs of the virus were detected within the spinach sample. This suggested that food matrices may play a role in protecting virus from inactivation. Taken together, these results demonstrated that MNV-1 was more resistant to gamma irradiation than most bacterial pathogens. Less than 2 log virus reduction was achieved in all three fresh produce samples at the FDA approved dose (4kGy). Therefore, it seems impractical to use gamma irradiation as a means to inactivate virus in fresh produce.

VSV, an enveloped virus, is more sensitive to gamma irradiation. We compared the stability of VSV (an enveloped virus) and MNV-1 (a non-enveloped virus) to gamma irradiation. As shown in Fig.2, VSV was much more sensitive to gamma irradiation than MNV-1. Both VSV and MNV-1 decreased in virus titer as irradiation dose increased in intensity. However, VSV was reduced at a greater concentration starting from 5.6kGy. At this dose, a 3.3 log virus reduction was observed in VSV, while only 1.7 log virus reduction was observed for MNV-1. At the dose of 16.8kGy, VSV was completely inactivated as confirmed by plaque assays. But, there were still 3.4 log virus survivors of MNV-1. Actually, an additional 5.6kGy was required to completely inactive MNV-1.

We further tested the stability of MNV-1 and VSV in different buffers. Briefly, MNV-1 and VSV were diluted into four different solutions (water, PBS, DMEM and DMEM plus 10%

FBS) and treated by two different irradiation doses (2.8 and 5.6 kGy), respectively. As shown in Fig.3A, MNV-1 was stable in all four solutions when treated by gamma irradiation. There was no significant difference in virus reduction among these solutions ($P>0.05$). Approximately less than 1.0 and 2.0 log virus reductions were achieved at 2.8 and 5.6 kGy, respectively. In sharp contrast, the stability of VSV in these four solutions was highly different (Fig.3B). Specifically, VSV was highly susceptible to gamma irradiation when it was diluted in water and PBS. At 2.8 kGy, 2.7 log virus reductions were observed. At 5.6 kGy, 6.3 log virus reductions were observed. However, when VSV was diluted in DMEM and DMEM plus 10% FBS, only 1.1-2.5 log and 1.3-2.1 log virus reductions were observed at irradiation dose of 2.8 and 5.6 kGy, respectively. These results demonstrated that the matrix of solution does not affect the stability of MNV-1, but it has a dramatic impact on the stability of VSV. Again, these data demonstrated that VSV was much more susceptible to gamma irradiation than MNV-1.

Gamma irradiation degrades viral proteins. To gain mechanistic insight of viral inactivation, we selected three specific irradiation doses, 2.8kGy, 5.6kGy, and 22.4kGy. We chose 2.8kGy and 5.6kGy because less than 1 log and 2.4 log virus reductions were observed, respectively. A mixture of infectious and non-infectious virus particles would be present at these two doses. We chose 22.4kGy because viruses were completely inactivated, which allowed us to precisely determine what happens to virus particles and viral components when no infectious virus particles remained.

It would be lethal to the virus if viral proteins become damaged by gamma irradiation. The structure of MNV-1 is relatively simple. The surface of MNV-1 possesses a highly stable viral capsid protein VP1 that surrounds and protects the viral genomic RNA (33, 63). The

structure of VSV is significantly different with MNV-1. The viral genomic RNA is completely encapsulated by the nucleocapsid (N) protein, forming the N-RNA complex (36, 37). In addition, the viral RNA-dependent RNA polymerase complex (large polymerase protein L and the phosphoprotein P) is tightly bound to N-RNA which results in the formation of the RNP complex. This RNP complex is further surrounded by a matrix (M) protein and viral envelope. The viral glycoprotein (G) is anchored in the envelope. To gain mechanistic insight of viral inactivation by gamma irradiation, we first determined whether viral proteins were degraded. Briefly, 20 µg of highly purified MNV-1 and VSV were treated by gamma irradiation at three different doses: 2.8, 5.6, and 22.4kGy. After treatment, 2 µg of either treated or untreated samples were analyzed by SDS-PAGE followed by Commassie blue staining. As shown in Fig.4, the abundance of MNV-1 and VSV proteins gradually decreased when irradiation dose increased. For MNV-1, the major capsid protein VP1 (molecular weight approximately 58kDa) was visualized on SDS-PAGE (Fig.4A). After 2.8 and 5.6kGy treatments, the abundance of VP1 proteins decreased to 50% and 30% compared to untreated control, respectively (Fig.4A). At 22.4kGy, the VP1 protein was undetectable by SDS-PAGE analysis, suggesting that MNV-1 VP1 protein was completely degraded (Fig.4B). For VSV, five structural proteins, L, G, P, N and M, were observed on SDS-PAGE. However, each viral protein exhibited different sensitivity to gamma irradiation. VSV polymerase complex (L and P proteins) and G protein were highly sensitive to gamma irradiation and rapidly degraded after 2.8 and 5.6kGy doses of irradiation (Fig.4C). These viral proteins were completely degraded after 22.4 kGy treatment (Fig.4D). VSV M proteins displayed moderate sensitivity. There were approximately 30%, 20% and 5% of M protein visualized after treatment of 2.8kGy, 5.6kGy, 22.4kGy doses respectively. The N protein was the most resistant VSV protein to irradiation treatment. There were 45 % and 35% N protein

348 detected after 2.8 kGy and 5.6kGy doses, respectively (Fig.4C). Even after 22.4kGy treatment,
349 there was approximately 25% of N protein remaining (Fig. 4D). In summary, these data clearly
350 demonstrated that gamma irradiation degraded viral proteins. However, the sensitivity of each
351 viral protein to irradiation varied.

352 Subsequently, we determined whether the remaining proteins from 2.8 and 5.6 kGy
353 treatments were antigenic. To address this question, Western blot was performed using antibody
354 against MNV-1 VP1 or VSV G protein. As shown in Fig.5, the abundance of MNV-1 VP1 and
355 VSV G proteins from Western blot essentially correlated with the amount of proteins from
356 Commassie blue staining. Specifically, 50% and 30% of MNV-1 VP1 protein were detected by
357 Commassie blue staining after 2.8 and 5.6kGy treatment, respectively. Based on the size of the
358 bands observed in Western blot, 65% and 40% of MNV-1 VP1 protein were detected (Fig.5A).
359 For VSV, 15% and 10% of G protein were detected by Commassie blue staining after 2.8 and
360 5.6kGy treatment (Fig.4C). In Western blot, 20% and 13% of G protein were detected (Fig.5B).
361 Therefore, these results demonstrated that the undegraded viral proteins still reacted with
362 monoclonal and polyclonal antibodies and perhaps retained correct primary amino acid
363 sequences.
364

365 **Gamma irradiation damages virus particles.** To determine whether gamma irradiation directly
366 damages the virus particles, we analyzed the virus particles by electron microscopy. For the
367 untreated control, MNV-1 particles were small spherical structured virions of 30-38 nm in
368 diameter (Fig.6A). After 2.8 and 5.6kGy irradiation treatment, the number of virus particles was
369 significantly reduced (Fig. 6B and C). Clearly, this was due to the fact that the viral capsid

370 protein was degraded by gamma irradiation. At 22.4kGy treatment dose, we failed to detect any
371 small round structured virions (Fig.6D), suggesting that virus particles were completely
372 disrupted. There were some debris materials observed by EM, which perhaps was the degraded
373 viral protein or mixture of degraded protein and RNA. For VSV, the virion is a bullet-shaped
374 particle that is approximately 70 nm in diameter and 140 nm in length (Fig. 6E). After 2.8kGy
375 treatment, VSV exhibited some morphological changes (Fig. 6F). Some virions became rounder
376 and more ambiguous rather than the traditional bullet-shaped virions (Fig. 6F). In the 5.6kGy
377 treated sample, less intact virions were observed. A large number of VSV virions were damaged
378 and thus did not retain their original geometry (Fig. 6G). The viral envelope was not uniform and
379 less defined. After 22.4kGy treatment, there was no intact bullet-shaped VSV particle observed
380 under EM. Instead, a large concentration of damaged viruses clumped together (Fig. 6H). These
381 clumped materials contained a few particles with severe damage and physical distortions. The
382 viral envelopes were lost and viral N-RNA complex was spilling out of the damaged particles.
383 Taken together, these results demonstrated that gamma irradiation damaged virus particles which
384 in turn resulted in the inactivation of the viruses.
385
386 **Gamma irradiation degrades viral genomic RNA**. MNV-1 is a positive-sense RNA virus
387 whereas VSV is a non-segmented negative sense RNA virus. It would be lethal to the virus if
388 viral RNA genome becomes damaged and/or degraded. Briefly, highly purified MNV-1 and
389 VSV were treated with gamma irradiation at three doses: 2.8, 5.6, and 22.4kGy. After treatment,
390 RNA was extracted from each sample followed by RT-PCR to amplify the MNV-1 VP1 and
391 VSV N gene, and the products were visualized by gel electrophoresis on 1% agarose gel. As
392 shown in Fig.7A, the VP1 gene was amplified in RNA samples extracted from MNV-1 treated

by 2.8 and 5.6kGy irradiation doses although the abundance of VP1 gene decreased compared with the untreated controls. This was not surprising since there were significant amounts of virus survivors at both doses. However, VP1 gene was not detectable in RNA sample from MNV-1 stock treated by 22.4kGy irradiation in which MNV-1 was completely inactivated (Fig.7B), suggesting that MNV-1 genomic RNA was completely degraded after this dosage of irradiation exposure. Similarly, the amount of VSV N gene decreased when VSV was treated by 2.8 and 5.6kGy irradiation (Fig.7C). Interestingly, a significant amount of VSV N gene was still detected in RNA sample from the VSV that was treated for 22.4kGy (Fig.7D). It should be emphasized that VSV was completely inactivated at dose of 16.8 kGy (Fig.2). Since VSV genomic RNA was completely encapsidated by N protein, intact RNA may still present if N protein was not completely degraded. Indeed, approximately 25% of VSV N protein remained after 22.4 kGy treatment (Fig.4D). Therefore, VSV genome was still detectable by RT-PCR at this irradiation dose.

Next, we directly treated the viral genomic RNA by gamma irradiation. Briefly, viral genomic RNA was extracted from MNV-1 and VSV, and treated by three irradiation doses (2.8, 5.6, and 11.2 kGy), followed by RT-PCR. At dose of 5.6 and 11.2 kGy, MNV-1 and VSV genomic RNA was undetectable by RT-PCR, respectively (data not shown). Taken together, these data demonstrated that gamma irradiation degraded viral genomic RNA.

Gamma irradiation damages human norovirus virus-like particles (VLPs). Unfortunately, the study of the survival of human norovirus is hampered because it cannot be grown in cell culture systems (19). However, it is known that expression of human norovirus VP1 protein in insect cells results in self-assembly of VLPs that are structurally and antigenically identical to

native virions (6, 14, 30). Therefore, VLPs have been used as a tool to understand the biology of human norovirus (6. 14, 30). In fact, our above experiments demonstrated that the disruption of viral proteins and structure is one of the mechanisms of virus inactivation. Thus, we wanted to demonstrate the sensitivity of human norovirus VLPs to gamma irradiation. Briefly, the capsid gene VP1 of human norovirus strain HS66 was cloned into the baculovirus expression system, expressed in insect cells, and the VLPs were purified as described in Materials and Methods. The purified VLPs were negatively stained with ammonium molybdate, and analyzed by EM. As shown in Fig. 8A, the expressed VP1 protein formed small round structured particles which were identical to native human norovirus virions. Consistent with previous observations (6), two sizes of particles were found in EM analysis (Fig.8A). The size of the larger particles was between 30-38 nm, and the smaller size was between 18-20 nm. To determine whether gamma irradiation damages the capsid, human norovirus VLPs were treated at three doses of irradiation. At a dose of 2.8 kGy, VLPs were clumped together and the morphology of VLPs was altered (Fig.8B). At a dose of 5.6 kGy, we observed a large concentration of protein debris and the structure of VLPs disappeared (Fig.8C). After 22.4kGy irradiation, we could not find any small spherical structured VLPs (Fig.8D), suggesting that VLPs were completely disrupted by gamma irradiation.

To demonstrate whether gamma irradiation degrades the capsid protein, the irradiated VLPs were analyzed by SDS-PAGE. For the untreated control, two protein bands with molecular weights of 58 and 55 kDa were observed (Fig.9A, lane 2). The 58kDa protein represented the native full-length VP1 protein and the 55 kDa protein was the cleaved form of VP1 protein (cVP1). This is consistent with the previous observation that baculovirus expressed VP1 is cleaved in insect cells (6, 30). After irradiation, the abundance of both protein bands was significantly diminished. Approximately 40% and 25% of human norovirus VP1 protein

439 remained after treatment at doses of 2.8 and 5.8 kGy (Fig.9A, lanes 2-3), and VP1 protein was
440 not visualized at all after 22.4 kGy of irradiation (Fig.9A, lane 4). For MNV-1 VP1 protein, 50%
441 and 30% VP1 remained after 2.8 and 5.8 kGy treatment. Furthermore, similar to MNV-1 VP1,
442 the remaining human norovirus VP1 from irradiation still reacted with the polyclonal antibody
443 (Fig.9B), suggesting that the undegraded protein was still antigenic. Fig. 9C showed the direct
444 comparison of the stability of the capsid proteins of MNV-1 and human norovirus by gamma
445 irradiation. Indeed, there was no significant difference between these two capsid proteins
446 ($P>0.05$). Therefore, it seems that the irradiation dose that degraded MNV-1 VP1 may be
447 sufficient to degrade human norovirus VP1.
448
449
450 **Discussion**
451
452 Irradiation was patented for food preservation in 1905 in France. Since then, irradiation
453 has been found as an effective food processing technology to eliminate bacteria, insects, fungi,
454 and pests, and poses no significant risks to human health or the environment (5, 17, 34, 45, 59).
455 However, the feasibility of gamma irradiation to eliminate viruses in foods is relatively less
456 understood. In this study, we found that MNV-1, a human norovirus surrogate, is resistant to
457 gamma irradiation. Only 1.7-2.4 logs virus reduction was observed in fresh produce samples at
458 irradiation dose of 5.6 kGy. Although VSV, an enveloped virus, is more susceptible than MNV-
459 1, only 3.3 log reductions were achieved at irradiation dose of 5.6 kGy in DMEM. Overall,
460 viruses are much more resistant to irradiation than bacterial pathogens. Furthermore, we found
461 that gamma irradiation degraded viral structural proteins, genetic materials, and damaged viral

462 particles which resulted in viral inactivation. Although gamma irradiation seems impractical to
463 eliminate the virus contaminants in fresh produce by the FDA approved irradiation dose limits,
464 this technology may be practical to inactivate viruses in other foods and to sterilize medical
465 devices, ophthalmic solutions, pharmaceuticals, tissue culture sera, animal waste, and municipal
466 sewage (17, 28, 29, 55, 57, 58).
467

468 **Gamma irradiation is not effective in eliminating human norovirus surrogate in fresh**
469 **produce.** A large amount of outbreak data showed that fresh produce has become one of the
470 major vehicles of transmitting foodborne viruses, particularly human norovirus (2, 8, 26, 39, 53).
471 Recent evidence showed that human norovirus not only tightly binds to fresh produce but also
472 can become internalized (50, 61, 62). Traditional sanitization strategy is thus not effective in
473 removing human norovirus from produce, particularly for those internalized virions. To improve
474 the safety of fresh produce, the FDA has approved food irradiation up to dose of 4 kGy to
475 inactivate bacterial pathogens. It has been demonstrated that irradiation dose of 4-5 kGy almost
476 completely inactivated major foodborne bacterial pathogens such as *Escherichia coli* O157:H7,
477 *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella typhimurium, and* mycotoxin
478 producing *Aspergillus* spp. and *Fusarium* spp.(5, 17, 34, 45, 52, 59).

479 Our initial objective was to determine whether foodborne viruses can be inactivated at the
480 approved irradiation dose. Unfortunately, according to our results, a 5.6kGy dose only eliminated
481 MNV-1 in a range between 2.2-2.4 logs in the produce samples. Thus, the irradiation dose that
482 normally would eliminate all pathogenic bacteria would not be sufficient to inactivate the
483 norovirus surrogate. Previously, gamma irradiation of other two human norovirus surrogates,

484 feline calicivirus (FCV) and canine calicivirus (CaCV) has been reported (16). Surprisingly, a 3
485 log virus reduction was observed by a very low dose of gamma irradiation (0.5kGy for FCV and
486 0.3 kGy for CaCV) (16). Based on our results, MNV-1 is much more resistant than FCV and
487 CaCV to gamma irradiation. Irradiation of other food and waterborne viruses such as hepatitis A
488 virus (HAV) (9), rotavirus (41), and poliovirus (27), has been reported. It was found that the D_{10}
489 values (dose required to reduce infectivity by 1 log 10) for HAV in lettuce and strawberries were
490 2.72$\pm$0.05 and 2.97$\pm$0.18 kGy, respectively (9), while the D_{10} value for gamma irradiating HAV
491 in clams and oysters was 2 kGy (41). It was also reported that D_{10} value for rotavirus required
492 2.4 kGy (41). Poliovirus inoculated to fish fillets required a dose of 6 kGy to achieve a 2-log
493 reduction (27). Overall, major food and waterborne viruses are highly resistant to gamma
494 irradiation. It seems impractical to utilize gamma irradiation to target viruses in fresh produce for
495 the food processing industry at the approved doses. However, it is possible that food irradiation
496 can be used for inactivating viruses in other food products. According to FDA Regulations 21
497 CFR 179.26, a medium dose of irradiation (up to 10kGy) can be used for killing pathogenic and
498 spoilage microorganisms within fresh fish, mushrooms, and frozen poultry. Medium dose
499 irradiation is also employed for reducing the cooking time of dehydrated vegetables and
500 extending the shelf life of food products. High dose (10-50kGy) irradiation is reserved for
501 sterilization of spices, herbs, seasonings, meats and other prepared foods in combination with
502 heat in order to inactivate enzymes.
503
504 **Mechanism of viral inactivation by gamma irradiation.** In early studies, it is thought that the
505 damage of genetic material was the primary mechanism of microorganism inactivation by
506 gamma irradiation (55, 57, 58). Gamma rays can directly "hit" the genetic material and indirectly

507 react with the nucleic acid via free radicals generated when gamma rays strike water molecules,
508 which results in single or double strand breaks, cross linkage breaks, and nucleotide degradation.
509 Consistent with this, the amount of MNV-1 VP1 gene decreased when the irradiation dose
510 increased. For MNV-1, genomic RNA was undetectable when the virus was treated by 22.4kGy
511 irradiation, suggesting that RNA was broken down. However, genetic material may not be
512 completely degraded when a virus becomes inactivated. For example, VSV was completely
513 killed after 16.8kGy dose, but a significant amount of VSV N gene was amplified even after 22.4
514 kGy treatment (Fig.7D), a dose well beyond its inactivation limit. This was consistent with the
515 fact that there was a significant amount of viral N protein remaining (Fig. 4D), which acted as a
516 shield for the genetic material. It is also possible that RNA damage from irradiation was
517 fortuitously not in the segment that was amplified in RT-PCR.

518 In addition to RNA degradation, we found that gamma irradiation also damaged virion
519 structure and viral proteins. The number of intact virus particles and the concentration of viral
520 proteins gradually decreased when gamma irradiation dose increased. The damage caused by
521 gamma irradiation included breaking the viral envelope, disrupting the viral capsid, and
522 physically distorting virion geometry. Gamma irradiation may not only disrupt the covalent
523 bonds but also non-covalent interactions such as: hydrogen bonds, ionic bonds, van der Waals
524 forces, and hydrophobic interactions, which are responsible for the secondary, tertiary, and
525 quaternary structure of a protein. The full-length viral proteins were physically eroded without
526 observation of intermediate products such as small peptides or protein fragments. This suggested
527 that gamma irradiation not only disrupted quaternary and tertiary structure of a protein but also
528 broke down secondary structure and primary amino acid sequences. Although virus inactivation
529 is essentially a one-hit event, some viral proteins may entail many more hits at high gamma

530 irradiation doses. It was likely that these peptides, fragments, or amino acids were too small to be
531 resolved by SDS-PAGE. Interestingly, the remaining viral proteins after 2.8 and 5.6 kGy of
532 irradiation still reacted with antibody, suggesting that these proteins retained correct primary
533 amino acid sequences. Previously, it was shown that UV irradiation affected the functions of the
534 capsid proteins of HAV and FCV (46, 47). It will be interesting to determine whether UV also
535 affects the integrity of virion structure.

536 We found that VSV was much more sensitive to gamma irradiation than MNV-1. There
537 may be many factors contributing to irradiation sensitivity. First, the genome size of VSV (11
538 kb) is larger than that of MNV-1 (7.7 kb). Indeed, early studies suggest there is an inverse
539 relationship between inactivation dose and viral genome size (55, 57, 58). Second, enveloped
540 viruses are likely more sensitive to gamma irradiation since the envelope may be easily damaged
541 by irradiation. Third, VSV is structurally more complicated than that of MNV-1. VSV possesses
542 five structural proteins, and damage of any of these structural proteins would be lethal to the
543 virus. Finally, the size of virus particle may also contribute to the irradiation sensitivity. Larger
544 particles would more likely to be hit by gamma rays due to the larger exposed surface area.

545 A clearer understanding of the mechanism of viral inactivation would also guide us to
546 properly use gamma irradiation processing. Prior to our study, it is thought that damage of viral
547 genetic material is the major target for virus inactivation (28, 29, 55, 57, 58). Therefore, gamma
548 irradiation is widely used as means to prepare inactivated viral vaccines (12, 42). However, our
549 mechanistic studies strongly suggest that gamma irradiation is not an ideal tool for preparing
550 inactivated vaccines. First of all, a low dose of gamma irradiation is not able to completely
551 inactivate the viruses, which will compromise the safety of the vaccine. More importantly, a high

552 dose of gamma irradiation disrupts the structure of virus particle and physically reduces total
553 viral proteins including the immunogenic antigens (such as MNV-1 VP1 and VSV G) which are
554 responsible for triggering an effective immune response. Thus, the efficacy of the vaccine would
555 likely be impaired because the antigens would be damaged or severely diminished.
556
557 **Stability of the capsid proteins of MNV-1 and human norovirus to gamma irradiation.**
558 Because human norovirus cannot be grown in cell culture systems (19), no survival data are
559 currently available for this important pathogen that has dramatic impacts on food safety and
560 public health. Using human norovirus VLPs as a model, we demonstrated that the capsid protein
561 of human norovirus was degraded by gamma irradiation. For calicivirus, it is widely accepted
562 that VLPs are structurally, morphologically, and antigenically identical to native virion (6, 7, 14,
563 30, 49). Three-dimension structure imaging revealed that human norovirus VLPs are composed
564 of 90 dimers of the capsid protein, each of which forms an arch-like capsomere (14, 49). Such a
565 structure is highly stable and thus can protect genomic RNA. For MNV-1, there was 50% and
566 30% of VP1 protein remaining after 2.8 and 5.6 kGy of irradiation, respectively. However, there
567 was 40% and 25% of human norovirus VP1 remaining after 2.8 and 5.6 kGy of irradiation,
568 respectively. Similar to MNV-1, human norovirus VLPs were completely degraded at 22.4 kGy
569 of irradiation. This suggests that the capsid proteins of human norovirus and MNV-1 may have
570 an equivalent stability by gamma irradiation. EM analysis showed that gamma irradiation altered
571 the morphology of VLPs which resulted in the disruption of VLPs. Although our study was
572 based on human norovirus VLPs, it is the first evidence that the degradation kinetics of human
573 norovirus capsid by gamma irradiation is similar to that of MNV-1. It is worthy to emphasize
574 that human norovirus also encodes a minor capsid protein, VP2, a 18-kDa protein that plays an

575 important role in stabilizing the major capsid protein (VP1) by preventing the virus particle from
576 degradation and disassembly (7). The native human norovirus possesses both VP1 and VP2,
577 which is likely more stable than VLPs that only contain VP1 alone. Taking account of this factor,
578 it is possible that human norovirus may be even more stable than MNV-1 to gamma irradiation.

579 In summary, we demonstrated that human norovirus surrogate is resistant to gamma
580 irradiation. We provided first evidence that the capsid of human norovirus has an equivalent
581 stability compared to its surrogate. Furthermore, we provided new mechanistic insights to viral
582 inactivation by gamma irradiation. A better understanding of mechanism of viral inactivation
583 will guide the proper application of irradiation in industry.

584

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773 ## Figure legend

774 **FIG. 1. Gamma irradiation of MNV-1 in fresh produce and cell culture medium.** MNV-1
775 stock (10^8 PFU/ml) solutions were inoculated into spinach, lettuce, strawberry, and DMEM to
776 achieve an inoculation level of 10^7 PFU/g or 10^7 PFU/ ml. Prepared samples were irradiated up
777 to 22.4 kGy and were stomached for 2 min. Survival plot was determined by plaque assays. Data
778 points were averages of three replicates. Error bars represent ± 1 standard deviation.
779

780 **FIG. 2. Comparison of the sensitivity of MNV-1 and VSV to gamma irradiation.** MNV-1
781 stock (10^8 PFU/ml) and VSV stock (10^9 PFU/ml) were inoculated in DMEM and exposed to
782 irradiation up to 22.4kGy. Survival plot was determined by plaque assays. Data points were
783 averages of three replicates. Error bars represent ± 1 standard deviation.
784

785 **FIG.3. Stability of MNV-1 and VSV in different buffers treated by gamma irradiation.**

786 MNV-1 and VSV stocks were inoculated into four different buffers (water, PBS, DMEM, and
787 DMEM plus 10% FBS) at final concentration of 10^7 and 10^8 PFU/ml, respectively. The samples
788 were exposed to 2.8 and 5.6 kGy of irradiation. Virus survivors were determined by plaque
789 assays. Data points were averages of three replicates. (A) Stability of MNV-1 in different buffer.
790 (B) Stability of VSV in different buffer. The inactivation kinetic of VSV in water and PBS is
791 indistinguishable.
792

793 **FIG. 4. Gamma irradiation degrades MNV-1 and VSV structural proteins.** (A) SDS-PAGE
794 analysis of purified MNV-1 irradiated at 2.8kGy and 5.6kGy. Total viral proteins were analyzed

795 by 12% SDS-PAGE followed by commassie staining. VP1 = MNV-1 capsid protein. (B) SDS-
796 PAGE analysis of purified MNV-1 irradiated at 22.4 kGy. No VP1 protein was present after the
797 treatment. (C) SDS-PAGE analysis of purified VSV irradiated at 2.8kGy and 5.6kGy. Five
798 structural proteins of VSV, L, G, P, N and M proteins were visualized after commassie blue
799 staining. (D) SDS-PAGE analysis of purified VSV irradiated at 22.4 kGy. Only VSV N and M
800 proteins were visualized after the treatment.
801
802 **FIG. 5. Western blot analysis of MNV-1 capsid protein and VSV G protein after gamma**
803 **irradiation.** (A) Western blot analysis of MNV-1 capsid protein. Purified MNV-1 was
804 irradiated at doses of 2.8 and 5.6 kGy. Total proteins were separated by SDS-PAGE and
805 subjected to Western blot using rabbit anti-MNV VP1 polyclonal antibody. (B) Western blot
806 analysis of VSV G protein. Purified VSV was irradiated at doses of 2.8 and 5.6 kGy. Total
807 proteins were separated by SDS-PAGE and subjected to Western blot using monoclonal antibody
808 against VSV G protein.
809
810 **FIG. 6. Gamma irradiation damages MNV-1 and VSV.** Purified MNV-1 and VSV were
811 irradiated at doses of 2.8, 5.6 and 22.4 kGy. Treated and un-treated virus particles were
812 negatively stained with 1% ammonium molybdate and visualized by transmission electron
813 microscope. (A) Untreated MNV-1 virion. (B) MNV-1 particles treated by 2.8 kGy. (C) MNV-1
814 particles treated by 5.6 kGy. (D) MNV-1 particles treated by 22.4 kGy. (E) Untreated VSV
815 virion. (F) VSV particles treated by 2.8 kGy. (G) VSV particles treated by 5.6 kGy. (H) VSV
816 particles treated by 22.4 kGy.
817

818 **FIG. 7. RT-PCR analysis of MNV-1 and VSV after gamma irradiation.** (A) Detection of
819 VP1 gene from MNV-1 irradiated at 2.8 and 5.6kGy. Viral genomic RNA was extracted from
820 either treated or untreated MNV-1. The VP1 gene of MNV-1 was amplified by one-step RT-
821 PCR, and PCR products were visualized on 1% agarose gel electrophoresis. (B) Detection of
822 VP1 gene from MNV-1 irradiated at 22.4kGy. (C) Detection of N gene from VSV irradiated at
823 2.8 and 5.6 kGy. Viral genomic RNA was extracted from either treated or untreated VSV. The
824 VSV N gene was amplified by one-step RT-PCR. (D) Detection of N gene from VSV irradiated
825 at 22.4kGy.
826
827 **FIG. 8. Gamma irradiation damages human norovirus VLPs**. Human norovirus VLPs were
828 expressed and purified from insect cells using baculovirus expression system. The VLPs were
829 irradiated at three doses, 2.8, 5.6, and 22.4 kGy. Treated and un-treated VLPs were negatively
830 stained with 1% ammonium molybdate and visualized by transmission electron microscope. (A)
831 Untreated human norovirus VLPs. (B) VLPs treated by 2.8 kGy. (C) VLPs treated by 5.6 kGy.
832 (D) VLPs treated by 22.4 kGy.
833
834 **FIG.9. Gamma irradiation degrades the capsid protein of human norovirus.** (A)
835 Visualization of human norovirus capsid protein by 12% SDS-PAGE. The purified VLPs were
836 irradiated at 2.8, 5.6 and 22.4 kGy. Total viral proteins were analyzed by 12% SDS-PAGE
837 followed by commassie staining. VP1 = human norovirus capsid protein. cVP1= cleaved VP1
838 protein. (B) Western blot analysis of human norovirus VP1 protein. Identical samples from panel
839 A were separated by SDS-PAGE and subjected to Western blot using a polyclonal antibody
840 against VP1 protein. (C) Comparison of the stability of the capsid proteins of MNV-1 and human

norovirus by gamma irradiation. Two µg of MNV-1 and human norovirus VLPs were treated at 2.8, 5.6 and 22.4 kGy. Total proteins were separated by SDS-PAGE followed by commassie staining. The remaining proteins from gamma irradiation were quantified by ImageQuant TL software. Data points were averages of three replicates.

Figure 1

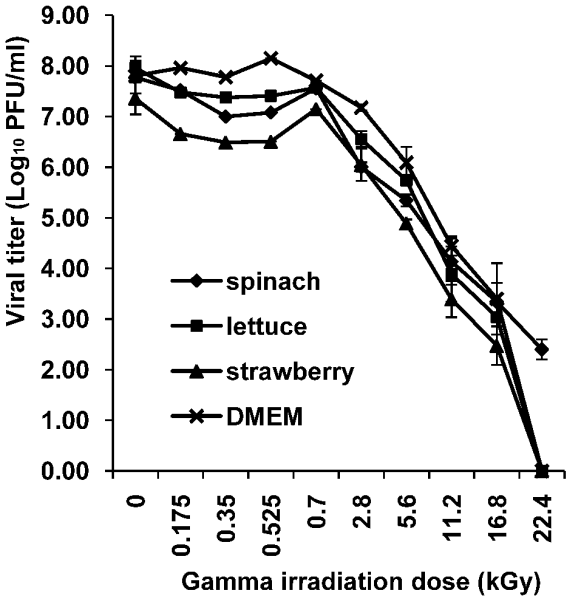
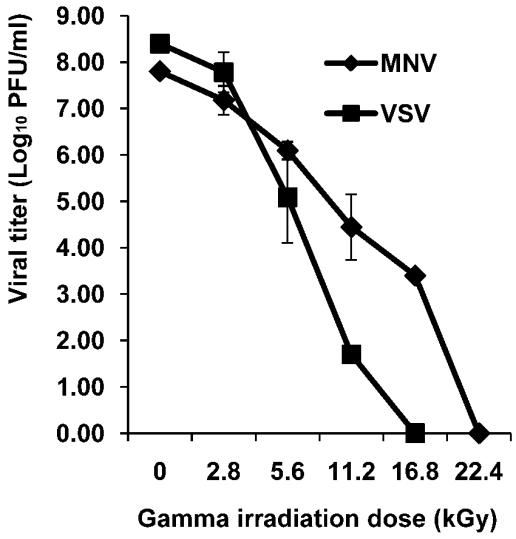


Figure 2



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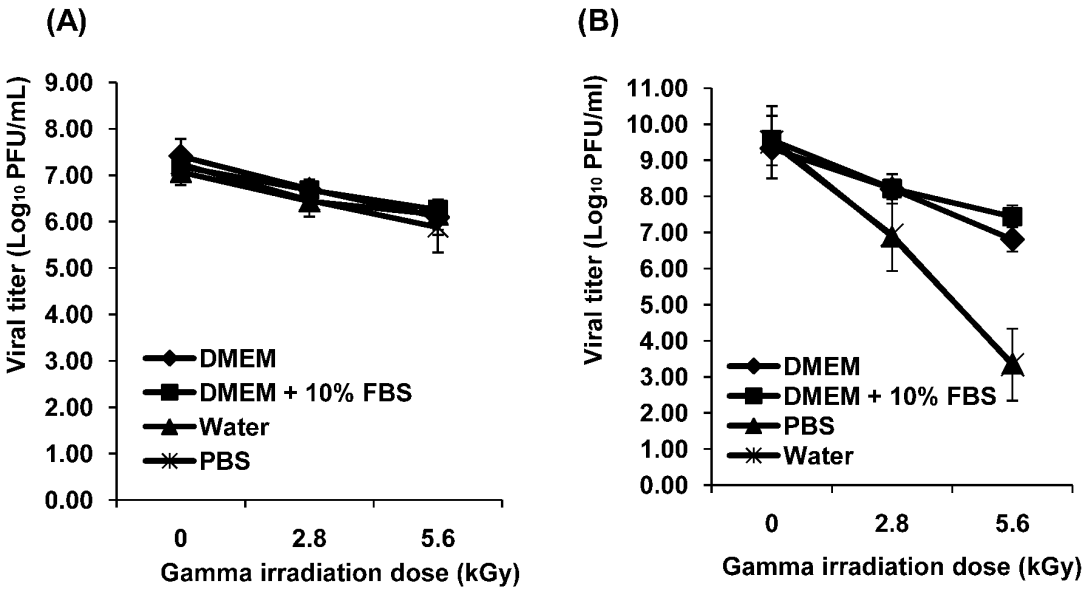

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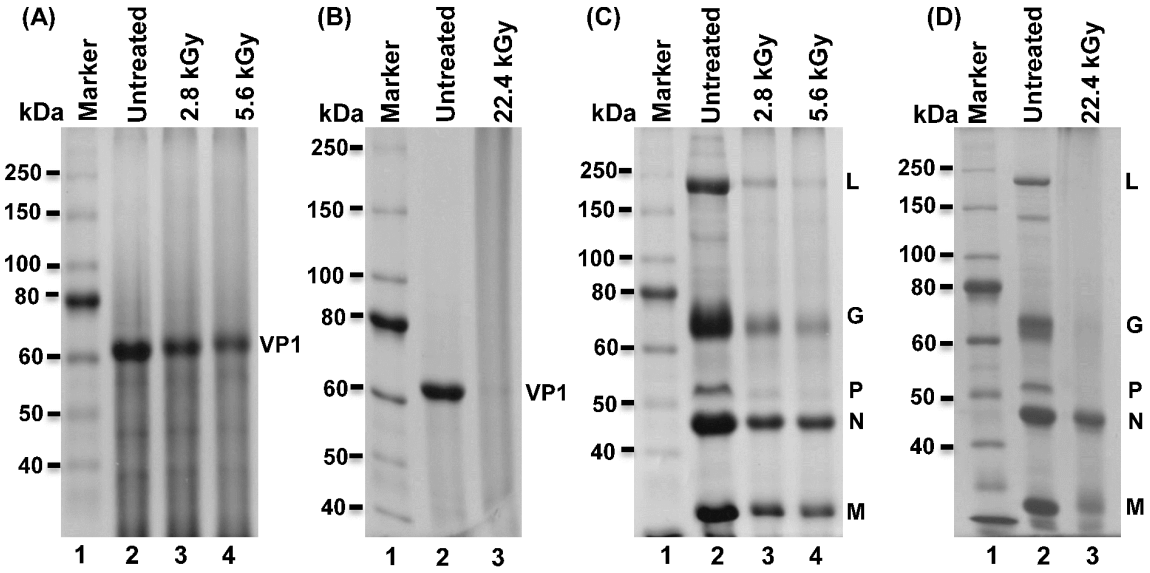

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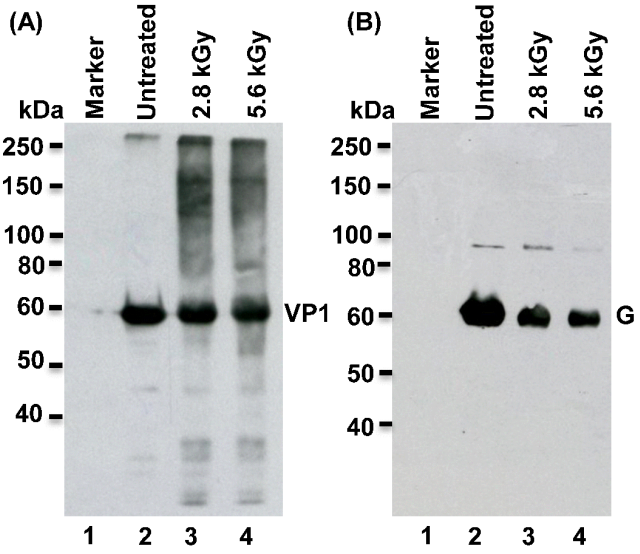
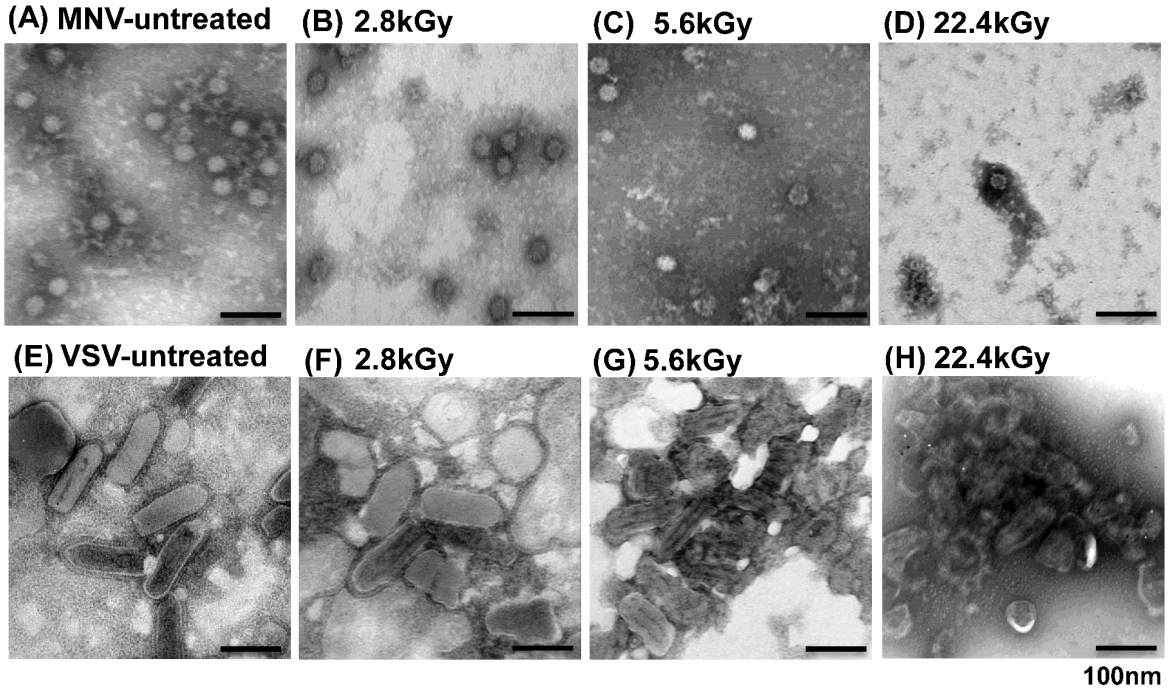


Figure 6



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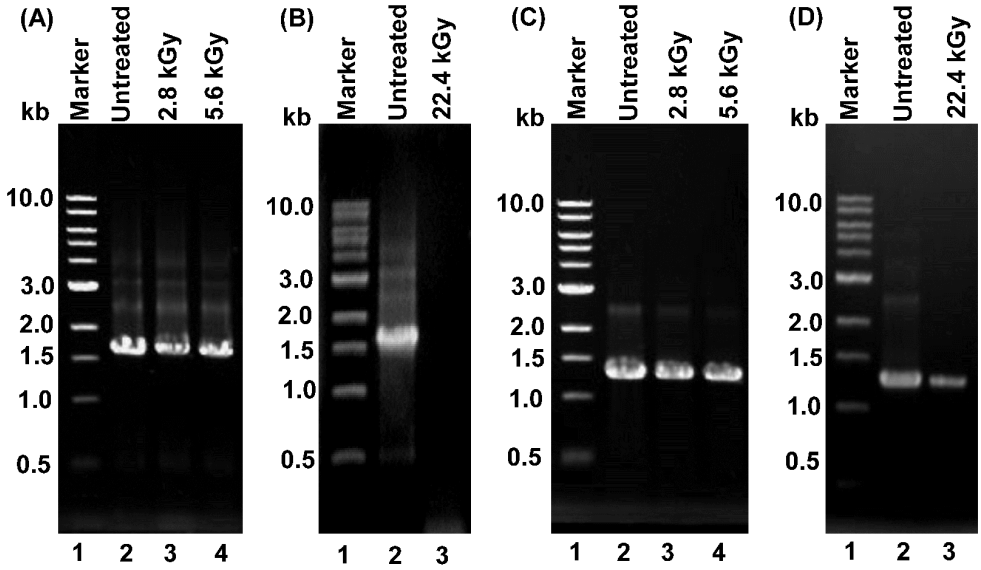

Figure 8

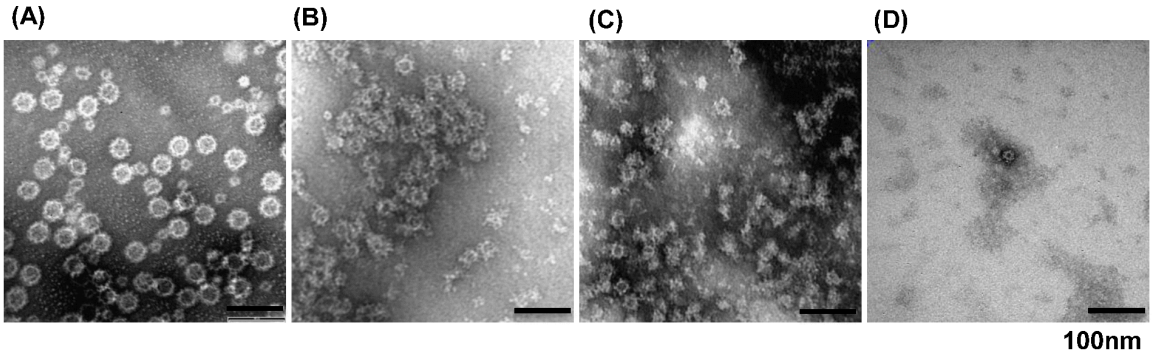

Figure 9